

University of Groningen

In vitro hemocompatibility testing

Blok, Sjoerd Leendert Johannes; Engels, Gerwin Erik; van Oeveren, Willem

Published in:
Biointerphases

DOI:
[10.1116/1.4941850](https://doi.org/10.1116/1.4941850)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Blok, S. L. J., Engels, G. E., & van Oeveren, W. (2016). In vitro hemocompatibility testing: The importance of fresh blood. *Biointerphases*, 11(2), [029802]. <https://doi.org/10.1116/1.4941850>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

In vitro hemocompatibility testing: The importance of fresh blood

Sjoerd Leendert Johannes Blok, Gerwin Erik Engels, and Willem van Oeveren

Citation: [Biointerphases](#) **11**, 029802 (2016); doi: 10.1116/1.4941850

View online: <https://doi.org/10.1116/1.4941850>

View Table of Contents: <http://avs.scitation.org/toc/bip/11/2>

Published by the [American Vacuum Society](#)

Articles you may be interested in

[In vitro blood flow model with physiological wall shear stress for hemocompatibility testing—An example of coronary stent testing](#)

[Biointerphases](#) **11**, 031004 (2016); 10.1116/1.4958979

[Thrombogenicity and hemocompatibility of biomaterials](#)

[Biointerphases](#) **11**, 029601 (2016); 10.1116/1.4938557

[Are there sufficient standards for the in vitro hemocompatibility testing of biomaterials?](#)

[Biointerphases](#) **8**, 33 (2013); 10.1186/1559-4106-8-33

[Preface: In Focus Issue on Blood–Biomaterial Interactions](#)

[Biointerphases](#) **11**, 029501 (2016); 10.1116/1.4954646

[Nanomaterials and nanoparticles: Sources and toxicity](#)

[Biointerphases](#) **2**, MR17 (2007); 10.1116/1.2815690

[Perspectives on the use of biomaterials to store platelets for transfusion](#)

[Biointerphases](#) **11**, 029701 (2016); 10.1116/1.4952450

Spectra
Simplified

Plot, compare, and validate
your data with just a click

eSpectra:
surface science

SEE HOW IT WORKS

In vitro hemocompatibility testing: The importance of fresh blood

Sjoerd Leendert Johannes Blok and Gerwin Erik Engels

HaemoScan BV, Stavangerweg 23-23, 9723 JC Groningen, The Netherlands

Willem van Oeveren^{a)}

HaemoScan BV, Stavangerweg 23-23, 9723 JC Groningen, The Netherlands

and Department of Cardiothoracic Surgery, University of Groningen,

University Medical Center Groningen, 9713 GZ Groningen, The Netherlands

(Received 23 December 2015; accepted 2 February 2016; published 12 February 2016)

The use of unactivated blood for hemocompatibility testing is essential to obtain reliable results. Here, the authors study the influence of heparinized whole blood storage time and temperature on blood activation and evaluate the importance of initiating hemocompatibility tests within 4 h of blood collection. Blood from healthy volunteers was collected and analyzed with minimal delay, after 30 min and after 60 min of storage at room temperature, 30 or 37 °C. In addition, blood was analyzed after 1, 2, or 4 h of storage at room temperature. Platelet count, mean platelet volume, platelet binding capacity to collagen and thromboxane B2 were measured to assess platelet function, complement complex C5b-9 and elastase were measured to assess activation of the inflammatory response system, and thrombin-antithrombin III was measured to assess activation of the coagulation system. Furthermore, free hemoglobin was measured in platelet poor plasma as an indicator for red blood cell damage. The authors found that storage at 30 °C significantly increased platelet and coagulation activity after 60 min and storage at 37 °C significantly increased platelet, coagulation, and white blood cell activity after 60 min. Storage at room temperature significantly decreased platelet binding to collagen after 4 h and increased platelet activity after 1 h onward and white blood cell activity after 4 h. Their results show that short-term storage of heparinized whole blood significantly influences biomarkers over time, especially at 30 and 37 °C compared to room temperature. However, blood stored at room temperature for 4 h is also affected. In particular, platelet function and white blood cell activity are significantly influenced after 4 h of stationary storage at room temperature; therefore, the authors propose that hemocompatibility tests should be initiated well within 4 h of blood collection, preferably within 2 h. © 2016 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>). [<http://dx.doi.org/10.1116/1.4941850>]

I. INTRODUCTION

The use of medical devices in the cardiovascular system has increased the demand for evaluation of their effects on blood. Direct contact of biomaterials with blood results in the activation of platelets (PLTs), the coagulation system, complement cascades and white blood cells.¹ These cascades are also activated during blood collection and storage before hemocompatibility testing or before processing in the clinical laboratory. Even with the use of inert storage containers, blood activation can occur due to the absence of endothelium-derived inhibitors. Due to irreversible activation of these cascades, a loss of function can ultimately result in false negative or positive data during analysis. Therefore, the stability and functionality of blood samples are continuously discussed in the clinical community, especially the lesion of stored platelets.^{2,3}

In order to obtain unbiased hemocompatibility results, it is important to use unactivated blood which is as similar to the clinical setting as possible.¹ By using a clinical dose of heparin (1.5 IU/ml), a dose often used during cardiovascular procedures, blood coagulation can be prevented without

inactivating the cascades,¹ which are needed for hemocompatibility testing.

Braune *et al.* stated that the total test duration should not exceed 4 h to ensure an appropriate function of blood cells and blood plasma proteins,⁴ since it is observed that blood activation starts shortly after blood collection. Thus, blood storage time and temperature between collection and initiation of hemocompatibility tests could significantly influence results of hemocompatibility testing. The ISO 10993-Part 4 standard advises that hemocompatibility tests should be performed with minimal delay, usually within 4 h of blood collection,⁵ without reference to valid data. Following this standard, several hemocompatibility studies described initiation of hemocompatibility tests within 4 h of blood collection.^{6,7} However, there are also numerous studies regarding hemocompatibility testing without any note on the analysis time. There are few studies describing the effects of short-term storage on biomarkers. A previous study described the influences of citrated whole blood- and platelet rich plasma (PRP) storage times on platelet- morphology and function and stated that platelet morphology and function, particularly platelet reactivity to adherent or soluble agonists changed rapidly outside the vascular system.⁸ Another study reported the stability of complement biomarkers in whole blood

^{a)}Electronic mail: w.vanoeveren@haemoscan.com

containing citrate or ethylenediaminetetraacetic acid (EDTA) and described that C4d, C3a, factor Bb, C5a and C5b-9 were stable at room temperature for up to 4 h and that thawing at 37 °C resulted in increased levels of complement markers in serum and citrated plasma but not in EDTA plasma.⁹ However, to our knowledge, there are no reports on short-term stability of the key determinants of hemocompatibility in heparinized whole blood.

The purpose of this study was to obtain further insight into the effects of storage time and temperature prior to hemocompatibility testing. Therefore, we analyzed platelet function, inflammatory response, coagulation and hemolysis of whole blood over time. Blood from healthy volunteers was collected and analyzed with minimal delay and after 30- and 60 min of storage at room temperature, 30 or 37 °C. From these data, it was concluded that storage at room temperature was most preferable. Subsequently, the experiment was repeated with storage times up to 4 h at room temperature.

II. MATERIALS AND METHODS

A. Experimental setup

During the first part of this study, blood was collected and stored at room temperature (21–25 °C), 30 or 37 °C for up to 60 min. Key determinants of hemocompatibility were analyzed and the effects of storage time and temperature were examined. These data clearly indicated that storage at room temperature was preferable. Therefore, in the second part of the study, whole blood was stored at room temperature for up to 4 h and the key determinants of hemocompatibility were analyzed and the effects of storage time were further examined. In addition, concentrations of the generated activation markers: Thromboxane B2 (TXB2), complement complex C5b-9, elastase, thrombin-antithrombin III (TAT III) complex and free hemoglobin (Hb) after stationary storage at room temperature for 4 h were compared with the concentrations generated in whole blood which had been *in vitro* circulated as previously described.¹⁰ The *in vitro* circulations (Hemobile, HaemoScan BV, Groningen, The Netherlands) were performed in polyvinyl chloride (PVC) circuits at 37 °C for 1 h.

B. Blood collection

For the analysis of storage up to 60 min at different temperatures, fresh human blood was collected by venipuncture with a 19 Gauge butterfly needle from six healthy adult volunteers (age 23–26) with a female to male ratio of 1:1 and anticoagulated with 1.5 IU heparin/ml (Leo Pharmaceutical Products BV, Weesp, The Netherlands). In addition, fresh human blood was collected and anticoagulated in the same manner from nine healthy adult volunteers (age 19–28) with a female to male ratio of 5:4 for storage up to 4 h at room temperature. The *in vitro* circulations were performed with fresh human whole blood from six healthy volunteers which was collected and anticoagulated as described above.

C. Storage

Heparinized whole blood was transferred to test tubes (Cellstar® Tubes, Greiner Bio-One GmbH, Frickenhausen, Germany) with minimal delay, and in the first part of the study, they were stored stationary at room temperature (21–25 °C), 30 or 37 °C for 30 or 60 min. Additionally, in the second part of the study, heparinized whole blood was stored in the same manner at room temperature for 1, 2, or 4 h.

D. Platelet function

Whole blood was centrifuged at 79×g for 5 min, and the supernatant was used as PRP of which PLTs were counted (cell counter Medonic CA 530, Medonic, Sweden). Platelet count and mean platelet volume (MPV) were analyzed (Medonic CA 530) on whole blood containing 5 mM EDTA (preventing any further blood activation) and PLT count was corrected for the EDTA dilution. The remaining EDTA containing whole blood was centrifuged at 13 400×g for 1 min, and the supernatant was used as platelet poor plasma (PPP).

Collagen is well known for its PLT binding capability; thus, binding of PLTs to collagen was used to assess PLT function. The capacity of PLTs to bind to collagen was analyzed in collagen-coated microtiter plates immediately after PRP was obtained. Collagen-coated microtiter plates were

TABLE I. Activation markers of whole blood stored at room temperature, 30 or 37 °C.

| Analyses | Room temperature | | | | 30 °C | | | 37 °C | | |
|------------------|------------------|---------------|---------------|-----------------------|---------------|---------------|-----------------------|---------------|---------------|-----------------------|
| | 0 min | 30 min | 60 min | <i>p</i> ^a | 30 min | 60 min | <i>p</i> ^a | 30 min | 60 min | <i>p</i> ^a |
| PLT (10E9/L) | 251 ± 64 | 235 ± 79 | 233 ± 56 | 0.583 | 239 ± 56 | 227 ± 57 | 0.400 | 231 ± 49 | 227 ± 42 | 0.307 |
| MPV (fl) | 9.28 ± 0.479 | 9.23 ± 0.709 | 9.20 ± 0.666 | 0.517 | 9.30 ± 0.583 | 9.07 ± 0.843 | 0.273 | 9.47 ± 0.662 | 9.18 ± 0.126 | 0.101 |
| PLT-Col (%) | 7.55 ± 3.77 | 6.86 ± 4.51 | 12.30 ± 6.44 | 0.173 | 7.66 ± 4.46 | 10.1 ± 5.83 | 0.379 | 7.80 ± 5.45 | 12.12 ± 8.64 | 0.292 |
| TXB2 (ng/ml) | 0.966 ± 1.33 | 2.48 ± 1.86 | 1.20 ± 0.410 | 0.721 | 3.12 ± 2.28 | 7.28 ± 3.93 | 0.025 | 1.71 ± 2.08 | 5.06 ± 2.71 | 0.044 |
| C5b-9 (ng/ml) | 197 ± 401 | 242 ± 522 | 192 ± 417 | 0.611 | 296 ± 640 | 214 ± 464 | 0.562 | 294 ± 636 | 217 ± 390 | 0.227 |
| Elastase (μg/ml) | 1.95 ± 1.16 | 1.85 ± 0.819 | 2.35 ± 1.41 | 0.668 | 2.54 ± 1.02 | 3.09 ± 1.64 | 0.187 | 2.10 ± 0.803 | 4.12 ± 1.85 | 0.001 |
| TAT III (μg/ml) | 1.35 ± 0.840 | 2.07 ± 2.54 | 2.19 ± 1.50 | 0.362 | 1.97 ± 2.71 | 5.29 ± 3.16 | 0.031 | 1.40 ± 1.26 | 4.88 ± 1.95 | 0.022 |
| Free Hb (%) | 0.085 ± 0.026 | 0.093 ± 0.074 | 0.055 ± 0.021 | 0.156 | 0.043 ± 0.026 | 0.055 ± 0.035 | 0.156 | 0.118 ± 0.054 | 0.100 ± 0.063 | 0.653 |

^aPaired samples t-test between baseline (0 min) and 60 min of storage. PLT, platelet count; MPV, mean platelet volume; PLT-Col, platelet binding to collagen; TXB2, thromboxane B2; C5b-9, complement complex C5b-9; TAT III, thrombin-antithrombin III complex; and Free Hb, free hemoglobin.

obtained by incubation of 96 wells flat-bottom microtiter plates (NUNC MaxiSorp®, Thermo Scientific, Inc., Roskilde, Denmark) with 100 µg/ml bovine type I collagen (Collagen solution from bovine skin, Sigma-Aldrich® Co. LLC., St. Louis, MO, USA) in 50 mM sodium carbonate, pH 9.6 at 2–8 °C overnight. After washing with phosphate-buffered saline, pH 7.4 (PBS), PLT binding was achieved by incubating PRP for 1 h at 37 °C. After washing with PBS, bound PLTs were analyzed based on the presence of acid phosphatase^{11,12} by incubating with 5 mM 4-nitrophenylphosphatase in citrate buffer +1% (v/v) Triton X-100, pH 5.4, on a shaker for 1 h. After addition of 1 M sodium hydroxide, conversion of the substrate was measured at 405 nm (PowerWave 200, Bio-Tek Instruments, Inc., Winooski, VT, USA). Optical density was related to PLT concentration using counted PLTs (Medonic CA 530) as a standard curve and the percentage of bound PLTs was determined.

Platelet activation leads to the activation of the arachidonic acid synthesis pathway to produce thromboxane A2.¹ Thromboxane A2 is highly unstable and rapidly converted to TXB2,¹³ i.e., TXB2 can be used as an indicator for PLT activation. Thromboxane B2 in PPP was analyzed by means of an enzyme immunoassay (Cayman Chemical Company, Michigan, USA), based on the competition between acetylcholinesterase labeled TXB2 and sample TXB2. Conversion of the substrate (Ellman's reagent) was measured at 415 nm (PowerWave 200).

E. Inflammatory response

Complement complex C5b-9 was analyzed as an indicator for complement activation. Complement complex C5b-9 in PPP was analyzed by means of an enzyme-linked immunosorbent assay (ELISA) based on a mouse anti human C5b-9 capture antibody (DAKO, Glostrup, Denmark) and a goat anti C5 detection antibody (Quidel, San Diego, CA, USA).

Elastase was analyzed as an indicator for white blood cell activation. Elastase in PPP was analyzed by means of ELISA based on a capture antibody against human elastase and a labeled antibody against alpha 1 antitrypsin (Affinity Biologicals, Inc., Ancaster, Canada).

F. Coagulation

Thrombin-antithrombin III complex was analyzed to determine thrombin formation, as an indicator for coagulation activity. Thrombin-antithrombin III complex in PPP was analyzed by means of ELISA, using antibodies of Cedarlane Laboratories, Ltd. (Hornby, Canada).

G. Hemolysis

Free Hb was used as an indicator for hemolysis, i.e., red blood cell damage, and was measured as described by Harboe¹⁴ (PowerWave 200). Percentage of hemolysis was determined by comparison with a 100% hemolysis sample.

H. Statistical analysis

For the statistical analysis of storage up to 4 h at room temperature, interdonor- and day-to-day variations were eliminated by normalizing all data to a percentage of the baseline (% baseline). Paired samples t-test was performed for all blood parameters to assess any significant difference ($p < 0.05$) between the different storage times and the

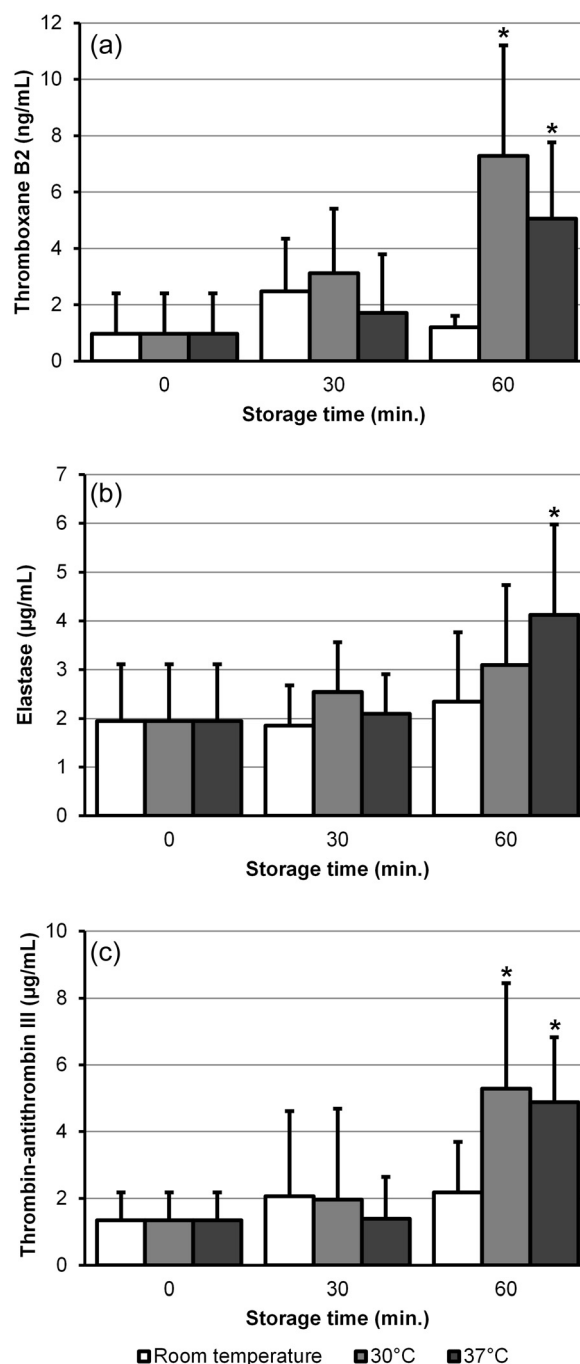


FIG. 1. Influences of whole blood storage at room temperature, 30 or 37 °C on thromboxane B2, elastase and thrombin-antithrombin III release in plasma during whole blood storage. (a) Thromboxane B2, (b) elastase and (c) thrombin-antithrombin III. * $p < 0.05$ compared with the baseline (0 min).

baseline. Normally distributed variables were reported as mean + one standard deviation.

III. RESULTS

A. Platelet function

Storage of whole blood at room temperature for up to 60 min did not change PLT count, MPV, PLT-collagen binding, or TXB2 concentrations over time (Table I). Storage of whole blood at 30 or 37 °C for up to 60 min did increase TXB2 concentrations over time (Table I, Fig. 1). Storage of whole blood at room temperature for up to 4 h did not affect PLT count or MPV over time (Table II). However, PLT-collagen binding decreased after 4 h (Table II, Fig. 2) and TXB2 concentrations increased after 1 h ($p = 0.005$), 2 h ($p \leq 0.000$) and 4 h ($p \leq 0.000$) (Table II, Fig. 2). Generated TXB2 of whole blood stored at room temperature for 4 h corresponded to 9% of the generated TXB2 in PVC circuits that circulated on the Hemobile (Table III).

B. Inflammatory response

Complement complex C5b-9 and elastase of whole blood stored at room temperature, 30 or 37 °C, for up to 60 min did not change over time (Table I). Storage of whole blood at 37 °C for up to 60 min increased elastase over time (Table I, Fig. 1). Generated complement complex C5b-9 of whole blood stored at room temperature for 4 h corresponded to 0.1% of the generated complement complex C5b-9 in PVC circuits that circulated on the Hemobile (Table III). However, complement C5b-9 did not change over time (Table II). Generated elastase of whole blood stored at room temperature for up to 4 h increased over time (Table II, Fig. 2) and after 4 h corresponded to 87% of the generated elastase in PVC circuits that circulated on the Hemobile (Table III).

C. Coagulation

Thrombin-antithrombin III complex of whole blood stored at room temperature for up to 60 min did not change over time (Table I). Thrombin-antithrombin III complex of whole blood stored at 30 or 37 °C did increase over time

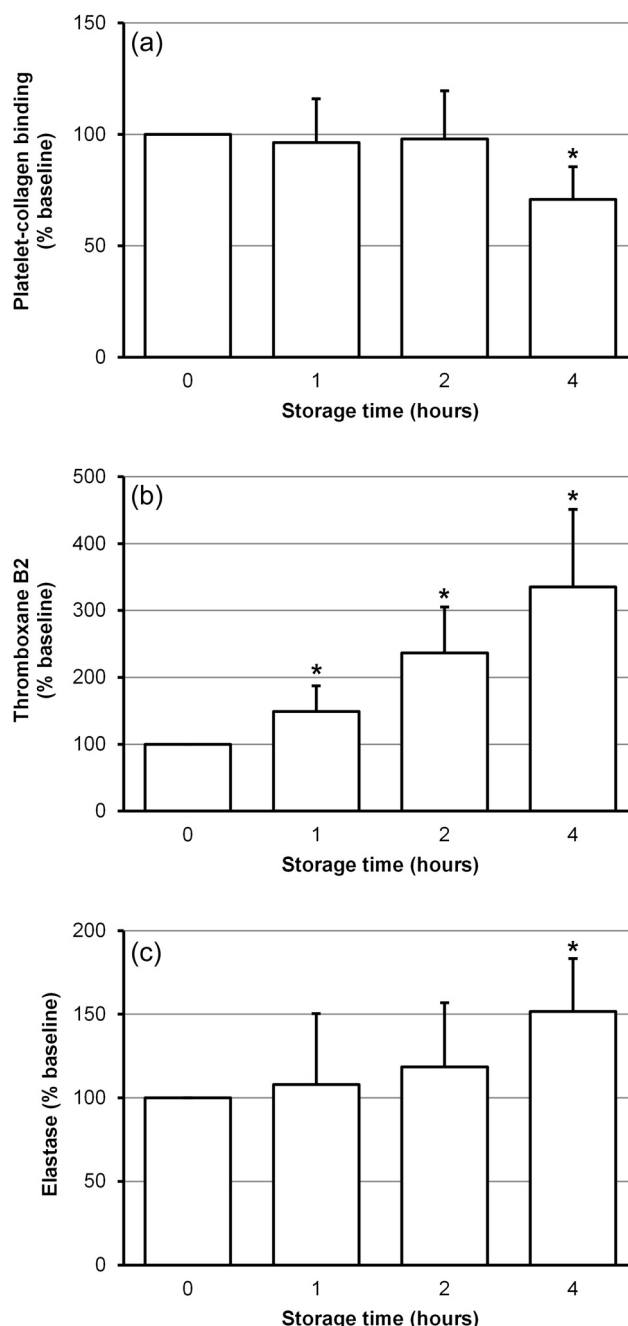


FIG. 2. Influences of whole blood storage at room temperature on platelet-collagen binding, thromboxane B2, and elastase. (a) Platelet-collagen binding, (b) thromboxane B2, and (c) elastase. * $p < 0.05$ compared with the baseline (0 h).

TABLE II. Activation markers of whole blood stored at room temperature for 1, 2, or 4-h.

| Analyses (% baseline) | Storage time (h) | | | p^a |
|---------------------------|------------------|-------------|-------------|-------|
| | 1 | 2 | 4 | |
| Platelet count | 97.1 ± 8.27 | 100 ± 8.9 | 99.2 ± 9.71 | 0.816 |
| Mean platelet volume | 101 ± 7.37 | 102 ± 6.5 | 99.8 ± 5.91 | 0.913 |
| Platelet-collagen binding | 96.3 ± 19.6 | 98.0 ± 21.5 | 70.8 ± 14.6 | 0.005 |
| Thromboxane B2 | 149 ± 38.5 | 236 ± 68.8 | 335 ± 116 | 0.000 |
| Complement complex C5b-9 | 95.8 ± 9.15 | 98.1 ± 8.08 | 102 ± 7.64 | 0.409 |
| Elastase | 108 ± 42.3 | 119 ± 38.2 | 152 ± 31.7 | 0.005 |
| Thrombin-antithrombin III | 86.3 ± 44.0 | 101 ± 55.4 | 220 ± 170 | 0.086 |
| Free hemoglobin | 80.0 ± 29.8 | 92.4 ± 36.1 | 94.8 ± 25.7 | 0.582 |

^aPaired samples t-test between baseline and 4 h of storage.

(Table I, Fig. 1). Generated TAT III complex of whole blood stored at room temperature for 4 h corresponded to 14% of the generated TAT III complex in PVC circuits that circulated on the Hemobile (Table III). However, TAT III complex of whole blood stored at room temperature for up to 4 h did not change over time (Table II).

D. Hemolysis

Free Hb of whole blood stored at room temperature, 30 or 37 °C, for up to 60 min did not change over time (Table I).

TABLE III. Generated activation markers of stored whole blood and *in vitro* circulations. PVC circuits: activation markers in whole blood before (baseline) and after (circulated) *in vitro* circulation of hemocompatible PVC circuits at 37 °C for 1 h; Storage: activation markers in whole blood before (baseline) and after (stored) storage at room temperature for 4 h; TXB2, thromboxane B2; C5b-9, complement complex C5b-9; TAT III, thrombin-antithrombin III complex; and Free Hb, free hemoglobin.

| Analyses | PVC circuits | | | Storage | | | Δ Storage/ Δ PVC (%) |
|------------------|-----------------|-------------------|--------------|-----------------|-------------------|--------------|------------------------------------|
| | Baseline | Circulated | Increase (%) | Baseline | Stored | Increase (%) | |
| TXB2 (ng/ml) | 966 \pm 1439 | 26408 \pm 12996 | 2632 | 475 \pm 98.8 | 1581 \pm 709 | 233 | 9 |
| C5b-9 (ng/ml) | 13.7 \pm 5.65 | 53.18 \pm 47.9 | 289 | 22.9 \pm 22.1 | 23.01 \pm 20.44 | 0.36 | 0.1 |
| Elastase (ng/ml) | 1949 \pm 1160 | 2947 \pm 1319 | 51.1 | 1949 \pm 363 | 2812 \pm 968 | 44.2 | 87 |
| TAT III (ng/ml) | 1349 \pm 839 | 8993 \pm 8634 | 566 | 659 \pm 1223 | 1179 \pm 2058 | 78.9 | 14 |
| Free Hb (%) | 0.10 \pm 0.03 | 0.25 \pm 0.14 | 159 | 0.03 \pm 0.03 | 0.04 \pm 0.03 | 60.4 | 38 |

Generated free Hb of whole blood stored at room temperature for 4 h corresponded to 38% of the generated free Hb in PVC circuits that circulated on the Hemobile (Table III). However, free Hb of whole blood stored at room temperature for up to 4 h did not change over time (Table II).

IV. DISCUSSION

The aim of this study was to describe the influence of storage time and temperature on the basic hemocompatibility variables: platelet function, inflammatory response, coagulation, and hemolysis in whole blood prior to hemocompatibility testing. First, we determined the effect of storage temperature—a low temperature is known to induce several platelet changes;¹⁵ therefore, we studied the temperature effects between room temperature and 37 °C. At room temperature, none of the parameters changed up to 60 min of storage time. However, storage at 30 or 37 °C increased blood activation after 60 min. These data clearly indicated that storage at room temperature was preferable. To obtain further insight into the influence of prolonged storage at room temperature, the parameters described earlier were also analyzed on whole blood stored at room temperature for 1, 2, or 4 h. Platelet count, MPV, complement complex C5b-9, TAT III complex, and free Hb were not influenced by storage at room temperature after 4 h as compared to baseline. However, PLT-collagen binding decreased during 4 h of storage at room temperature, TXB2 already increased after 1 h of storage at room temperature, and elastase increased during 4 h of storage at room temperature. Generated thromboxane B2, complement C5b-9 and TAT III complex in stored whole blood after 4 h were only 9%, 0.1%, and 14% of the generated concentrations in circulated PVC circuits, respectively. However, generated elastase and free Hb in stored whole blood after 4 h were 87% and 38% of the generated concentrations in circulated PVC circuits, respectively. However, as described earlier, free Hb did not change over time. Therefore, these results indicate that storage at room temperature does affect, in particular, platelets and white blood cells.

Blood activation can have a great impact on the reliability of blood tests, especially during hemocompatibility testing. For instance, the use of already activated blood during

hemocompatibility testing could result in an underestimation of blood activation initiated by the biomaterial of interest, due to possible exhaustion of activation products. On the other hand, it could intensify blood activation initiated by the biomaterial, resulting in an overestimation of blood activation. Braune *et al.* already described the influences of citrated whole blood and PRP storage times on platelet-morphology and function and stated that platelet morphology and function, particularly platelet reactivity to adherent or soluble agonists, changed rapidly outside the vascular system.⁸ Yang *et al.* reported the stability of complement biomarkers in whole blood containing citrate or EDTA.⁹ However, in serum without anticoagulant, increased complement activation was observed during 4 h of storage, which can be explained by the presence of complement cofactors: Ca²⁺ and Mg²⁺ ions. Likewise, in heparinized blood, the complement system is also prone to activation.

Our findings indicate that activation of platelets already takes place after 1 h of storage at room temperature. Furthermore, our findings indicate that reduction of platelet binding to collagen and activation of white blood cells takes place after 4 h. Therefore, our findings advocate hemocompatibility tests to be initiated well within 4 h of blood collection. However, several other arguments can be raised that allow the use of 4 h old blood. First, a baseline can be collected just prior to the use of stored blood. Second, during biocompatibility testing, reference materials should always be tested with the same blood as the material of interest to be able to eliminate donor variability. Third, the activation markers: TXB2, complement complex C5b-9, and TAT generated after 4 h of storage represent less than 15% of the generated concentrations after blood circulation in a PVC circuit, indicating that blood after 4 h of storage still has the capacity to respond to an activating surface. Furthermore, by using a positive reference during *in vitro* circulations, higher levels of activation markers can be achieved, resulting in an even lower percentage of storage activation compared to achievable activation during *in vitro* circulations.

Future studies may include further evaluation of 4 h stored blood used for *in vitro* circulation and because hemocompatibility tests are also frequently performed on citrate-anticoagulated blood, future studies may also include the

effects of storage time and temperature before initiation of hemocompatibility tests using citrate-anticoagulated blood.

V. CONCLUSION

While it has previously been stated that hemocompatibility of blood contacting medical devices should be thoroughly tested before certification,¹ requirements regarding the quality of blood used during hemocompatibility tests are still in their infancy. As part of the quality of blood used during hemocompatibility tests, information in relation to the effects of time and temperature between blood collection and hemocompatibility testing is still lacking. We observed that storage at room temperature for up to 4 h does affect in particular platelets and white blood cells. Although platelet activation and platelet function were moderately affected, white blood cell activation was similar to the activation measured in blood which had been circulated in PVC circuits. Stationary storage of whole blood at room temperature up to 4 h before initiation of hemocompatibility tests seems unfavorable. Therefore, we propose hemocompatibility tests to be initiated well within 4 h of blood collection, preferably within 2 h of blood collection.

- ¹W. van Oeveren, *Scientifica* **2013**, 392584 (2013).
- ²M. Shrivastava, *Transfus. Apheresis Sci.* **41**, 105 (2009).
- ³D. V. Devine and K. Serrano, *Clin. Lab. Med.* **30**, 475 (2010).
- ⁴S. Braune, M. Grunze, A. Straub, and F. Jung, *Biointerphases* **8**, 33 (2013).
- ⁵ISO 10993, *Biological Evaluation of Medical Devices—Part 4: Selection of Tests for Interactions with Blood* (International Organization for Standardization, Genève, 2002).
- ⁶T. C. Major, H. Handa, G. M. Annich, and R. H. Bartlett, *J. Biomater. Appl.* **29**, 479 (2014).
- ⁷P. Jansen, W. van Oeveren, A. Capel, and A. Carpentier, *Eur. J. Cardiothorac. Surg.* **41**, e166 (2012).
- ⁸S. Braune, M. Walter, F. Schulze, A. Lendlein, and F. Jung, *Clin. Hemorheol. Microcirc.* **58**, 159 (2014).
- ⁹S. Yang, M. McGookey, Y. Wang, S. R. Cataland, and H. M. Wu, *Am. J. Clin. Pathol.* **143**, 558 (2015).
- ¹⁰W. van Oeveren, I. F. Tiellu, and J. de Hart, *Int. J. Biomater.* **2012**, 673163 (2012).
- ¹¹P. Bellavite, G. Andrioli, P. Guzzo, P. Arigliano, S. Chirumbolo, F. Manzato, and C. Santonastaso, *Anal. Biochem.* **216**, 444 (1994).
- ¹²A. C. Eriksson and P. A. Whiss, *J. Pharmacol. Toxicol. Methods* **52**, 356 (2005).
- ¹³S. Saito, K. Yamasaki, S. Yamada, A. Matsumoto, T. Akatsu, N. Takahashi, Y. Shibasaki, T. Suda, and T. Fukuhara, *Bone Miner.* **12**, 15 (1991).
- ¹⁴M. Harboe, *Scand. J. Clin. Lab. Invest.* **11**, 66 (1959).
- ¹⁵M. G. Egidi, A. D'Alessandro, G. Mandarello, and L. Zolla, *Blood Transfus.* **8**, 73 (2010).